The Role of Nonspecific Serum Factors in the Antibody Response to the ΦX 174 Bacteriophage

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ABSTRACT

The role of the "antibody cofactor" and of other heat-labile serum components (complement) in the neutralization of the ΦX 174 bacteriophage by means of specific antibodies was studied. Sera of white mice, guinea-pigs and rabbits obtained mainly early after phage administration were investigated. The character of antibodies was estimated from their sensitivity to 2-mercaptoethanol or else by chromatography on Sephadex G-200. Sera from the first days after the administration of the phage containing mostly type 19S antibodies, and sera from later periods after the administration containing mostly type 7S antibodies, were tested. (Some evidence was also obtained about the formation of slowly sedimenting antibodies sensitive to 2-mercaptoethanol in the rabbit.) With a single exception the tested sera showed no significant decrease of the neutralization activity after 30-mins. heating at 56° C or at 60° C and no increase of the neutralization power could be observed after the application of homologous or normal mouse serum. It is concluded that the heat-labile components of normal sera including the complement and the "antibody cofactor" play no role in specific phage neutralization.

It is known that the neutralization of various viruses can by potentiated by adding normal serum to immune antiserum, in particular if the antiserum has been heated or kept for a longer time (Allen, Finkelstein & Sulkin, 1958). It was usually assumed that the enhancing factor was identical with complement but it was found here that in antisera against influenza viruses the enhancing factor is the so-called "antibody cofactor" which is a serum component quite different from complement (Styk, Kočišková & Hána, 1964).

It was the purpose of the present work to find out whether the "antibody cofactor" or another heat-labile component of normal and immune sera takes part in the neutralization of the ΦX 174 bacteriophage by specific antibodies. For this reason two indicators were examined: (1) The phage-neutralizing capacity of unheated immune sera was compared with those heated for 30 min. at 56 or 60° C. (2) Experiments were made to determine whether an increase would occur in the neutralizing capacity of heated immune sera on adding unheated normal serum. White mice, guinea-pigs and rabbits were used for the immunization experiments, for testing the potentiation of the neutralizing effect, normal mouse serum was used as well as a serum of a homologous animal species (guinea-pig or rabbit). Since the antibody cofactor exhibits its activity most pronouncedly in the investigation of "early" anti-influenza sera (Styk, 1962a) the anti-phage sera from the early periods after antigen application were mainly examined. The character of the antibodies in these sera (19S—7S) was also studied,

especially with a view to the sensitivity to 2-mercaptoethanol (Deutsch & Morton, 1957; Uhr & Finkelstein, 1963) and in some cases by determining the molecular size of antibodies by means of Sephadex G-200 chromatography (Gelotte, Flodin & Killander, 1962; Flodin & Killander, 1962).

MATERIALS AND METHODS

Phage. Lysate of the ΦX 174 bacteriophage in aerated broth culture of *Escherichia coli C* was used throughout. Its titre was 5×10^9 PFU/ml.

Immunization. Random-bred white mice weighing about 25 g. were given 1.0 ml. phage preparation i.p. The guinea-pigs were of two types: a group of young guinea-pigs weighing about 300 g. were given 1.0 ml. preparation each intracardially; a group of large adult guinea-pigs weighing about 600 g. were given 2.0 ml. preparation intracardially. Rabbits weighing about 2500 g. were given 5.0 ml. of the preparation intravenously; a part of the rabbit group were given another identical dose after 5 days. Revaccination was carried out after 44 days in the same way. In all cases an undiluted preparation was used. Mouse blood was obtained with a capillary from the retro-orbital venous plexus; guinea-pig and rabbit blood by cardiac puncture. Mouse sera were mixed, guinea-pig and rabbit sera were investigated individually. Before examination the sera were kept at —50° C.

Serological investigation. Ten-fold dilutions of heated or unheated immune sera were prepared in buffered saline. For investigating the enhancing effect of normal sera the corresponding dilution of heated serum was mixed with the same volume of normal unheated serum diluted with saline 1 : 10 (in some cases 1 : 5). For examining the effect of 2-mercaptoethanol (2-ME) the compound was added to a final concentration of 0.1M to each corresponding dilution of immune serum. (In the controls the corresponding volume of saline was added.) In all these cases the samples after mixing were incubated for 30 min., in a 37° C bath. Antibody titration took place in the presence of 2-ME. The same procedure was used for examining the fractions separated on Sephadex G-200; in these cases undiluted samples were also examined. Chromatography on Sephadex G-200 was carried out as described earlier (Hána, Styk & Schramek, 1963). The separation yielded three well resolved peaks containing, in sequence of running off the column, macroglobulins, 7S-gamma globulins and proteins resembling albumins in their size.

Determination of the neutralization titre of serum (or fraction). One ml. serum after appropriate treatment was mixed with 1 ml. phage diluted to contain 200 PFU/ml. After a 60-minute incubation at 37° C, 1 ml. of this mixture was plated onto a Petri dish with *E. coli C* as indicator. (For details see Adams, 1959). The titre of the serum (fraction) was calculated from the decrease in infectivity of the phage under the influence of the antiserum. We proceeded from the assumption that within certain limits the logarithm of the surviving fraction is proportional to serum concentration. The antiserum titre represents the reciprocal of the final dilution of serum in the reaction mixture which is capable of neutralizing 50% infectious phage particles within a one-hour incubation. A similar procedure for evaluating the titre was used by Svehag and Mandel (1962). The significance of titre differences was evaluated on the basis of the number of plaques in titration based on the tables of the plaque test statistics (Lorenz, 1962).

RESULTS

Blood was withdrawn from all experimental animals before beginning the immunization experiments. The serum of none of these animals contained substances that would neutralize the ΦX 174 bacteriophage. It was thus possible to evaluate the specific response to a sub-

sequent application of the phage to animals as well as to use these normal sera for enhancing the neutralization effect of heated antisera.

Table 1 presents the results of examining some selected sera obtained 5—12 days after inoculation of the bacteriophage. On comparing columns A and C it can be seen that in no case did we observe any significant decrease of the neutralizing titre after heating. In the case of the rabbit serum No. 21, which was obtained 12 days after immunization, there is a significant difference at the 5% level between the values before and after heating; in all other sera the differences are not significant. After adding normal serum (mouse or homologous) to heated antiserum no significant increase in the neutralization activity was observed in any case (cf. column C with D or E). The differences found in some sera are not significant at $p = 5\%$ level.

Since a 30-min. heating at 56° C may be insufficient for destroying the cofactor activity in guinea-pig serum (Styk, 1962b) some antisera were examined after heating for 30 min. at 60° C. The result was analogous to that in Table 1.

The antibody response in the individual guinea-pigs varied considerably in the height of the neutralization titre. For example 7 days after the injection of the antigen the antibody titres of guinea-pigs No. 11, 12 and 13 were 1020, 88 and 450, respectively. Irrespective of the titre no enhancing effect of normal serum components was found here.

Table 1. The effect of heating, 2-mercaptoethanol (2-ME) and normal serum on the neutralizing capacity (neutralization titres) of different antisera to ΦX 174.

Serum No.	Days after immunization	Unheated sera		Sera heated 30 mins. at 56° C			
		A (saline)	B (2-ME)	C saline	D (NMS)	E (NHS)	F (2-ME)
Mouse 1	7	100	23	110	72		<5
	12	110	92	92	130		31
Mouse 2	5	43	<5	40	47		<5
Guinea-pig 11	5	660	20	760	800	850	23
Guinea-pig 12	7	88	8	94	91	98	6
Rabbit 21	5	410	<5	330	310	380	< 5
	12	3800	200	2300	2800	3100	<50

NMS = normal mouse serum added 1 : 10
NHS = normal homologous serum added (guinea-pig or rabbit) 1 : 10

Table 2. Chromatography of immune sera obtained from rabbit No. 21 at different periods of immunization, on Sephadex G-200. Distribution of antibodies and their sensitivity to treatment with 2-ME (neutralization titres)

Days after immunization	Original serum		19S**		7S**	
	untreated	2-ME	untreated	2-ME	untreated	2-ME
5	410	0	84	0.7	1.5	1.0
12	3800	200	590	5	60	22
28	2900	2900	120	5	110	100
5 — rev.*	22000	12000	350	12	2400	720

* Serum obtained 5 days after revaccination carried out 44 days after first administration of the phage.
** Fraction from the top of the first (macroglobulin) or second (7S-gamma-globulin) peak after chromatography on Sephadex G-200.

Another negative result was obtained in the experiments aimed at showing the possible role of heat-labile serum components by following the kinetics of the phage neutralization. No significant difference was found to exist between the inactivation constants of (a) native, (b) heated and (c) heated plus fresh normal serum added.

The heat-labile components played no role when comparing heated and unheated fractions obtained after separation of antisera on Sephadex G-200. Thus, for example, the titre of the top fraction from the macroglobulin peak obtained after chromatography of early rabbit antiserum No. 21 (taken 5 days after immunization) did not decrease at all. As may be seen from Table 2 the serum contained practically exclusively antibodies of the 19S type distributed after chromatography on Sephadex G-200 in the macroglobulin peak. The further course of formation of 19S and 7S antibodies in rabbit No. 21 is shown in Table 2. (Table 2 shows only unheated sera or samples after chromatography).

Attention should be paid to the decrease of the titre following application of 2-mercaptoethanol in fractions from the 7S-gamma-globulin peak found 12 days after primoimmunization and 5 days after revaccination. In the former serum the decrease of the titre after application of 2-ME is significant at the 5% probability level; in the latter the decrease of the titre lies at the border of 5% probability. Separation of proteins from these two sera on Sephadex G-200 is represented in Fig. 1 which also shows fractions taken for determination of antibody titre and of sensitivity toward 2-ME. In every case we were dealing here with a mixture from two tubes belonging to the same peak. Similarly in another rabbit to whom two phage doses were administered at a 5-day interval the 7S-gamma-globulin peak (from serum 5 days after the 2nd injection) was found to contain antibodies sensitive to 2-ME: their titre decreased after applying 2-ME from more than 132 to 56 which is significant at the 1% level of probability.

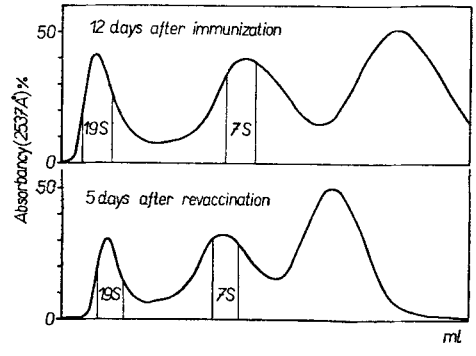

Fig. 1. Separation of proteins of two immune rabbit sera anti ΦX 174 on Sephadex G-200. Fractions are shown in which the content of antibodies and their sensitivity to 2-ME was studied (cf. Table 2).

DISCUSSION

Three species of experimental animals were used for the experiments: white mice, rabbits and guinea-pigs. White mice were chosen as a species in which the effect of the serum cofactor to anti-influenza antibodies is most striking; rabbits serve as one of the most common objects of immunological studies; guinea-pigs because they contain the highest level of the complement or of complement components. In the last-named case we could partly proceed from the findings of Uhr and Finkelstein (1963) who described the formation of rapidly and slowly sedimenting antibodies against the ΦX 174 phage in guinea-pigs; however, the authors did not study the possible effect of heat-labile serum components on the neutralizing capacity of the antiserum.

In none of the antisera tested in our experiments did we find any substantial decrease of the neutralizing capacity after heating or an increase of this capacity after adding normal mouse or homologous serum. This holds both for sera obtained during the first days after administration of the antigen where an

enhancing effect of fresh serum would be most expected and for sera withdrawn later during antibody formation. Unequivocal evidence was thus obtained that neither the "antibody cofactor" nor the complement system or another heat-labile component play a role in the neutralization of the ΦX 174 bacteriophage by specific antibodies.

It was not the aim of present work to investigate in any detail the character of the antibody response toward the ΦX 174 bacteriophage with regard to the formation of rapidly and slowly sedimenting antibodies. In this connection information is available from experiments with guinea-pigs (Uhr & Finkelstein, 1963) which, at the same time, points to the considerable variability of individual animals. These authors confirmed that, as in human serum macroglobulins (Deutsch & Morton, 1957), the rapidly sedimenting guinea-pig molecules of antibodies against ΦX 174 are depolymerized by 0.1 M 2-mercaptoethanol which is accompanied by a complete loss of antibody activity; on the other hand, the slowly sedimenting molecules of antibodies do not lose their activity after such treatment. Uhr and Finkelstein (1963) use sensitivity to 2-ME as the only criterion for classifying antibody molecules as rapidly or slowly sedimenting and designate them as 19S and 7S, respectively.

If this procedure is used for evaluating the sera examined here it can be seen immediately that Table 1 contains mainly sera with a predominance of 19S antibodies which is more or less pronounced. A greater content of 7S antibodies is found only in the mouse serum No. 1 obtained 12 days after antigen application. At the same time, the sensitivity to 2-ME is markedly increased in heated antisera. It must be borne in mind, however, that in two cases shown in Table 2 as well as in another rabbit serum sensitivity to 2-ME was exhibited even by antibodies contained in fractions from the second peak containing 7S-gamma-globulins. It is unlikely that we could be dealing here with an unsatisfactory separation of the 19S antibodies sensitive to 2-ME from the 7S antibodies. This is contradicted by curves of chromatographic fractionation of the above-mentioned sera indicating a fine resolution of proteins in the first and second peaks. We should rather consider the possibility that even in rabbits, slowly sedimenting antibodies sensitive to 2-ME can be formed as were found in sera of non-mammalian vertebrates (Uhr, Finkelstein & Franklin, 1962; Grey, 1963, and elsewhere) as well as their possible existence in mice as pointed out recently by Berlin (1963).

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РОЛЬ НЕСПЕЦИФИЧЕСКИХ СЫВОРОТОЧНЫХ ФАКТОРОВ ПРИ РЕАКЦИИ ОБРАЗОВАНИЯ АНТИТЕЛ ПРОТИВ БАКТЕРИОФАГА ФХ 174

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Изучалась возможная роль «кофактора антител», а также других термолабильных сывороточных компонентов (комплемент), при нейтрализации бактериофага ФХ174 специфическими антителами. Исследовались сыворотки белых мышей, морских свинок и кроликов, полученные, главным образом, на ранних сроках после введения фага. Характер антител определяли по чувствительности к 2-меркаптоэтанолу, а иногда также с помощью хроматографии на Sephadex G-200. Тестировались сыворотки, полученные в первые дни после применения фага, преимущественно содержавшие антитела типа 19S, и сыворотки, полученные в более поздний период, с более значительным содержанием антител 7S. (Были получены также сведения относительно образования медленно осаждающихся антител, чувствительных к 2-меркаптоэтанолу, у кролика.) За одним исключением, в исследуемых сыворотках не наблюдалось ни достоверного понижения активности нейтрализации после их 30-минутного прогревания при 56° С или 60° С, ни потенцирования нейтрализации после прибавления гомологичной (или мышиной) сыворотки. — Делается вывод, что термолабильные компоненты нормальных сывороток, включая комплемент и «кофактор антител», не играют роли в специфической нейтрализации фага.